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1 **Experimental climate effect on seasonal variability of**  
2 **polyphenol/phenoloxidase interplay along a narrow fen-bog ecological**  
3 **gradient in *Sphagnum fallax***

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23 Running title: phenol/phenoloxidase interplay in peatland

24

25 **Key words:** carbon cycle, climate warming, ecological gradient, open top chambers, peatland,  
26 phenoloxidases, polyphenols.

27

28 **Abstract**

29 Extracellular phenoloxidase enzymes play an important role in the stability of soil carbon  
30 storage by contributing to the cycling of complex recalcitrant phenolic compounds. Climate  
31 warming could affect peatland functioning through an alteration of polyphenol/phenoloxidase  
32 interplay, which could lead them to becoming weaker sinks of carbon. Here, we assessed the  
33 seasonal variability of total phenolics and phenoloxidases subjected to 2-3°C increase in air  
34 temperature using Open Top Chambers. The measurements were performed along a narrow  
35 fen-bog ecological gradient over one growing season. Climate warming had a weak effect on  
36 phenoloxidases, but reduced phenolics in both fen and bog areas. Multivariate analyses  
37 revealed a split between the areas and also showed that climate warming exacerbated the  
38 seasonal variability of polyphenols, culminating in a destabilization of the carbon cycle. A  
39 negative relationship between polyphenols and phenoloxidases was recorded in controls and  
40 climate treatments suggesting an inhibitory effect of phenolics on phenoloxidases. Any  
41 significant decrease of phenolics through repeatedly elevated temperature would greatly  
42 impact the ecosystem functioning and carbon cycle through an alteration of the interaction of  
43 polyphenols with microbial communities and the production of extracellular enzymes. Our  
44 climate treatments did not have the same impact along the fen-bog gradient and suggested that  
45 not all the peatland habitats would respond similarly to climate forcing.

46

47

## 48 **Introduction**

49 Boreal peatlands currently represent a terrestrial sink of carbon with approximately one-third  
50 of the world's organic carbon (390-455 Pg) (Gorham, 1991; Moore, 2002). The ability of  
51 peatlands to store atmospheric carbon resides in the long-term accumulation of partially  
52 decomposed organic matter. The accumulated peat is mainly dominated by remnants of  
53 mosses of the *Sphagnum* genus, highly enriched in recalcitrant organochemical compounds  
54 such as polyphenols (van Breemen, 1995; Verhoeven & Toth, 1995). Such compounds play a  
55 role both through a polyphenolic network linked to cell walls which could directly preserve  
56 *Sphagnum*-derived organic matter from degradation, and through the release of water soluble  
57 phenolics which directly interact with the surrounding environment (van Breemen, 1995;  
58 Verhoeven & Liefveld, 1997). Phenolics produced by *Sphagnum* have a potential inhibitory  
59 effect on fungal and bacterial activity and/or on enzymes involved in organic matter  
60 decomposition (Wetzel, 1992; Fenner *et al.*, 2005; Opelt *et al.*, 2007; Mellegard *et al.*, 2009).  
61 Among the diversity of enzymatic activities recorded in peat soils, only phenoloxidases –  
62 mainly produced by fungi – are involved in the polymerization, depolymerisation and  
63 transformation of both complex and simple phenolic compounds (Pind *et al.*, 1994; Thormann  
64 *et al.*, 2002; Fenner *et al.*, 2005; Baldrian, 2006; Sinsabaugh, 2010). However, acidic  
65 conditions, waterlogging and low soil temperatures that occur in peat soils were recognized to  
66 limit phenoloxidase activity (Pind *et al.*, 1994, Williams *et al.*, 2000; Freeman *et al.*, 2001a, b;  
67 Toberman *et al.*, 2008, 2010). Thus, carbon sequestration in peatlands is thought to partly  
68 result from a suppression of phenoloxidase activity (Freeman *et al.*, 2001a, 2004).

69 The expected increase of air temperatures in boreal regions is predicted to lead to a  
70 destabilization of peatland carbon stores (Smith *et al.*, 2004; Strack, 2008). Owing to the  
71 temperature regimes that currently constrain biological activities, climate warming may  
72 significantly impact the stability of the carbon cycle of peatlands by the breakdown of its

73 recalcitrant organic matter and thus act on “the enzymatic latch” (Freeman *et al.*, 2001a,  
74 2004). However, recent research on the effect of climate change on phenoloxidases highlight  
75 equivocal results in peatlands (Laiho, 2006; Fenner *et al.*, 2007; Toberman *et al.*, 2008, 2010).

76 In regions without permafrost the most fundamental distinction among peatland types  
77 is between bog and fen (Bridgham *et al.*, 1998, 2001; Rydin & Jeglum, 2006). Bogs and fens  
78 have been found to have different plant communities, hydrology, nutrient availability, and soil  
79 chemistry (Bridgahm *et al.*, 1998, 2001; Wheeler & Proctor, 2000; Rydin & Jeglum, 2006).  
80 Owing to these differences in biotic and abiotic settings, bogs and fens are likely to differ in  
81 their response to climate change, (Weltzin *et al.*, 2000, 2001, 2003). Recently, Jassey *et al.*  
82 (2011a) demonstrated that microorganisms (e.g. testate amoebae) and their interplay with  
83 polyphenols varied along a short fen-bog gradient. Accordingly, an understanding of how  
84 climate change modifies carbon cycling in peatlands by modifying the  
85 polyphenol/phenoloxidase interplay in different ecological setting is essential to assess the  
86 capacity of peatlands to continue to store carbon.

87 The aim of this study was to investigate the impact of experimental climate warming  
88 on seasonal variation of polyphenols, phenoloxidases and their interplay in different  
89 ecological settings. These factors were studied at two depths along the living *Sphagnum* shoot  
90 on a short ecological gradient from a transitional *Sphagnum*-dominated poor fen to a  
91 *Sphagnum* bog with more pronounced micro-topography. Temperatures were manipulated  
92 using open-top chambers placed on half of the sampling plots, and compared with control  
93 plots. We hypothesized that (1) seasonal variations of polyphenols, phenoloxidases and their  
94 interplay would be different between the structurally more complex *Sphagnum* “bog” habitat  
95 and the more uniform poor fen, and (2) the warming effect would alter the seasonal variations  
96 of these factors along the fen-bog gradient.

97 **Materials and methods**

98 Field site and vegetation

99 The study site is an undisturbed *Sphagnum*-dominated mire situated in the Jura Mountains  
100 (The Forbonnet peatland, France, 46°49'35''N, 6°10'20''E) at an altitude of 840 m a.s.l. Cold  
101 winters (on average -1.4°C) and mild summers (on average 14.6°C) characterize the site. The  
102 annual mean temperature measured at the site over a one-year period from 5<sup>th</sup> November 2008  
103 to 30<sup>th</sup> November 2009 was 6.5°C and the annual precipitation 1200 mm.

104 Samples of *Sphagnum fallax* were collected within homogeneous areas of *S. fallax*  
105 carpet across two adjacent areas selected in relation to their wetness, soil micro-topography,  
106 vegetation and assessment of sources and decay of organic matter according to Delarue *et al.*,  
107 (2011). The first sampling area (called “fen”) was a transitional *Sphagnum*-dominated poor  
108 fen with a relatively flat and homogeneous topography, characterized by a moss cover  
109 dominated by *S. fallax* and by the lack of *S. magellanicum*. Vascular plants such as  
110 *Eriophorum vaginatum*, *Vaccinium oxycoccus* and *Andromeda polifolia* were recorded in very  
111 low abundance. *Scheuchzeria palustris* and *Carex limosa* occurred outside of the studied  
112 plots. The second sampling area (called “bog”) was a *Sphagnum* bog directly adjacent to the  
113 fen area. Patterns of hummocks with *S. magellanicum*, *V. oxycoccus*, *E. vaginatum* and  
114 *Calluna vulgaris*, and hollows with lawns of *S. fallax*, *Carex rostrata* and *A. polifolia*  
115 characterized the sampling area. The terms “fen” and “bog” are used for simplicity and to  
116 denote the existence of a trophic and wetness gradient inferred from the vegetation.

117 Environmental manipulations and data collection

118 In each of the two sampling areas, six plots were selected in representative surfaces.  
119 Among the 12 sampling plots, the maximal distance between the two most distant plots was

120 ca. 30 m. In both sampling areas, 3 plots (replicates) were randomly assigned as controls and  
121 3 plots were assigned as climate warming treatment (begin April, 2008). An increase of air  
122 and soil temperatures was passively achieved by placing hexagonal ITEX open-top chambers  
123 (hereafter “OTC”) over the vegetation (Marion *et al.*, 1997). Since warming in OTC chambers  
124 also affects the top-soil humidity, we hereafter name this treatment “climate effect”.  
125 Hexagonal OTCs were 50 cm high, had a diameter of 1.8 m at the top and 2.5 m at the  
126 bottom, and were made of transparent polycarbonate. To reduce edge effects such as reduced  
127 precipitation in the chamber we used the OTC design described by Aerts *et al.* (2004) and  
128 Dorrepaal *et al.* (2004). In each plot, air temperature (10 cm above the *Sphagnum* surface) and  
129 soil temperature (7 cm below the *Sphagnum* surface) were recorded continuously every 30  
130 minutes using thermocouple probes and a datalogger (CR-1000 Campbell). Moreover, in each  
131 plot, pH, conductivity, water content of *Sphagnum* and the depth to the water table (DWT)  
132 were measured at each sampling campaign.

133 Every month from 25<sup>th</sup> May 2009 to 25<sup>th</sup> November 2009, samples of *S. fallax* were  
134 collected in each plot for the study of phenolic compounds, fungi-producing phenoloxidases  
135 and phenoloxidase activities around 10 permanent markers inserted in moss carpets. The goals  
136 of this sampling design were (1) to allow for multiple sampling at the site over time, and (2)  
137 to obtain a composite sample from each plot and avoid any bias due to spatial heterogeneity.  
138 *S. fallax* shoots were cut into two pieces (sampling depth): 0-3 cm (living “top segments”) and  
139 3-10 cm (early declining “bottom segments”) from the capitulum.

#### 140 Phenolic compounds quantification

141 Primarily bound (hereafter “bound phenolics”) and water-soluble phenolic (hereafter “free  
142 phenolics”) compounds were extracted from lyophilized mosses as described in Jassey *et al.*  
143 (2011a). Briefly, bound phenolic compounds were extracted using ethanol / distilled water

144 solution (80/20 v/v) and free phenolics using distilled water. Free and bound total phenolic  
145 contents were quantified with the Folin-Ciocalteu reagent and were expressed in mg  
146 equivalent gallic acid ( $A_{760}$ ) per gram of *Sphagnum* dry mass ( $\text{mg g}^{-1}$  DM).

#### 147 Quantification of culturable fungi-producing phenoloxidases

148 Culturable fungi-producing phenoloxidases were counted as described by Criquet *et al.*  
149 (2000). Two grams fresh weight of *Sphagnum* was powdered ( $< 0.5$  mm; SEB<sup>®</sup> *Optimo*  
150 compact mixer) and suspended in 250 mL of a 0.85% NaCl solution with 0.05% Tween 80.  
151 This mixture was agitated for 2h on a reciprocal shaker (120 rpm). The extract was diluted  
152 ( $10^{-1}$  to  $10^{-3}$ ) in NaCl (0.85%) solution and 0.1 mL of each dilution was used to inoculate a  
153 medium containing 5 g of malt (Sigma), 15 g of agar (Sigma), 50 mg of chloramphenicol  
154 (Sigma) and 0.5 mL of guaiacol (Sigma) per liter. The fungi-producing phenoloxidases were  
155 revealed by the red color of the environment related to the oxidation of guaiacol. Results are  
156 expressed in colony forming units per gram of *Sphagnum* dry mass ( $\text{CFU g}^{-1}$  DM).

#### 157 Phenoloxidase activities quantification

158 Phenoloxidase activities were quantified following the method described by Criquet *et*  
159 *al.* (1999). Phenoloxidases were extracted by adding in a Pyrex bottle 3 g of fresh weight of  
160 powdered *Sphagnum* with 50 mL of a 0.1 M  $\text{CaCl}_2$  solution with 0.05% Tween 80 and 20 g of  
161 polyvinylpyrrolidone. The samples were shaken at room temperature for 1h on a  
162 reciprocal shaker (120 rpm). The suspension of each extract was filtered through a double  
163 layer of gauze to remove floating debris and centrifuged at 10 000 g for 10 min at 4°C. Then  
164 the supernatant was filtrated through 1.2  $\mu\text{m}$  Whatman GF / D filters and concentrated for 24h  
165 in a cellulose-dialysis tube (Medicell International Ltd.) with a 10 kDa molecular mass cut-  
166 off, covered with polyethylene glycol (PEG, Sigma-Aldrich), until a final volume of 1/10 of  
167 the initial volume. Enzymatic activities were measured using a 96 well microtiter plate with



168 L-DOPA (10 mM). For each sample, 8 pseudo-replicate wells were included. Assay wells  
169 received 150 µl of extract. Phenoloxidase activities were measured by adding 100 µL of L-  
170 DOPA. For each sample, 8 pseudo-replicate wells containing 150 µl of boiled extract (2h at  
171 90°C) were performed as control. Then samples were incubated at 23°C and L-DOPA  
172 oxidation rates were monitored spectrophotometrically at 460 nm for 24h using a microstation  
173 plate reader (Bioadvance).

174 Enzymatic activities were calculated by subtracting the mean absorbance of control  
175 wells from the mean absorbance of extract wells and by using Beers Law. The molar  
176 absorbancy coefficient for the L-DOPA product 3-dihydroindole-5,6-quinone-2-carboxylate  
177 (dicq) ( $3.7 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ; Mason, 1948) was used and activities were expressed in enzymatic  
178 units (U) defined as one nmol of substrate oxidized per  $\text{h}^{-1}$  per g of dry m ass.

#### 179 Numerical analysis

180 To compare the general effects of the OTCs on environmental parameters during the 7 months  
181 of our study, daily average temperature, as well as minimum and maximum daily  
182 temperatures, pH and conductivity were calculated for spring (May-June), summer (late-June-  
183 September) and autumn (late-September-November). Then repeated measures ANOVA were  
184 computed among sampling areas to focus on the effect of OTCs on these factors with time as  
185 repeated measure (time = 3: spring, summer and autumn). The depth and climatic effect on  
186 phenolic compounds (free and bound), culturable fungi-producing phenoloxidases and  
187 phenoloxidase activities were also analysed using repeated measures ANOVA with time as  
188 repeated measure (time = 7: May-November). Each dataset was thereafter split by month to  
189 get one response matrix per month for each biological factor using one-way ANOVA. In  
190 parallel, correlations between free phenolics, fungi-producing phenoloxidases and  
191 phenoloxidase activity in controls and OTCs were determined along the fen-bog gradient

192 using general linear models (GLM) and one-way ANOVAs. The residuals from ANOVAs  
193 were tested for normality. Moreover, the coefficient of determination of each variable in the  
194 models (adjusted  $R^2$ ) was determined with an analysis of variance.

195 Redundancy analyses (RDA) were applied to *Sphagnum* related biochemical variables  
196 (polyphenols, phenoloxidasases, culturable fungi-producing phenoloxidasases) for each  
197 *Sphagnum* segment among the fen and the bog areas using climatic treatment (a binary  
198 variable with two levels: Control and OTC), *Sphagnum* moisture content and time (months  
199 coded as classes) as explanatory variables. The interactions between climatic treatment and  
200 *Sphagnum* moisture content were also included in the model. The significance of the model  
201 and of each explanatory variable included in the model was tested using 1,000 permutations  
202 (Gillet *et al.*, 2010). Partial RDAs were also computed after removing the time effect  
203 (months) from the ordination following the same method. Additionally, variation partitioning  
204 using RDA and adjusted  $R^2$  was applied to compare the respective effect of each explanatory  
205 variable alone (Peres-Neto *et al.* 2006).

206 Multiple factor analysis (MFA) was used to symmetrically link seven groups of  
207 descriptors split in seven sub-matrices: the two *Sphagnum* related biochemical matrices  
208 (phenolic compounds and phenoloxidase data sets), the two abiotic data sets describing  
209 physical (depth to water table, air and soil temperature, rainfall and *Sphagnum* moisture  
210 content) and chemical (conductivity and pH) environmental conditions, the climatic data set  
211 describing climate treatment (a binary variable with two levels: OTC coded with 1 and control  
212 with 0), and the two data sets describing the seasons (spring, summer or autumn coded as  
213 classes), and the sampling areas (fen or bog coded as classes). MFA was chosen because it  
214 allows the simultaneous coupling of several groups or subsets of variables defined on the  
215 same objects and to assess the general structure of the data (Escofier & Pagès, 1994). Briefly,  
216 MFA is basically a PCA applied to the whole set of variables in which each subset is

217 weighted, which balances inertia between the different groups and thus balances their  
218 influences. RV-coefficients (Pearson correlation coefficient, ranging from 0 to 1) were used to  
219 measure the similarities between two data matrices and were tested by permutations (Robert  
220 & Escoufier, 1976; Josse *et al.*, 2008). Euclidean distances of global PCA were used in MFA  
221 to perform cluster analysis according to the Ward method, and the resulting dendrogram was  
222 projected in the MFA ordination space. This allows discovering the main discontinuities  
223 among groups and/or sites described by all biotic and abiotic subsets of variables (Carlson *et*  
224 *al.*, 2010; Borcard *et al.*, 2011).

225 All multivariate analyses were performed with the software R 2.10.1 (R Development  
226 Core Team 2010) using the vegan (Oksanen *et al.*, 2010) and FactoMineR (Husson *et al.*,  
227 2009) packages.

228

## 229 **Results**

### 230 Seasonal variation of climate variables

231 In spring and summer (May to September), the OTCs significantly increased the daily  
232 maximum air temperature (an average of 3°C; ANOVA  $P < 0.01$ ) and the average air  
233 temperature (an average of 1°C; ANOVA  $P < 0.01$ ). Climate treatment also significantly  
234 affected the daily soil temperature in spring in the bog area (an average increase of 0.6°C;  
235 ANOVA  $P < 0.05$ ) and in summer in the fen area (an average increase of 0.8°C; ANOVA  $P <$   
236 0.05). No significant differences emerged for the minimum and maximum soil temperatures.  
237 In autumn, no significant effect of OTCs was recorded along the gradient for air and soil  
238 temperature. An indirect effect of climate treatment was also observed in *Sphagnum* mosses,  
239 since a significant decrease of *Sphagnum* water content in OTCs was recorded in summer

240 (August and September) in both *Sphagnum* segments in the bog area, and in top segments in  
241 the fen area (ANOVA  $P < 0.05$ . Fig. 1).

242         Rainfall significantly varied following the seasons with a decrease from June (156  
243 mm) to August, September and October (a monthly average of 72 mm) and an increase in  
244 November (231 mm). These variations were also reflected in the depth to water table.  
245 Following the seasons and climate treatments, average monthly pH did not significantly vary  
246 in both sampling areas (Table 1). Conversely, the conductivity increased from spring to  
247 autumn in both sampling areas, with significant differences between controls and OTCs in  
248 summer (bog area,  $P = 0.05$ ) and in autumn (fen area,  $P = 0.01$ ).

#### 249 Climate effect on phenolic compounds and seasonal variations

250 Regardless of seasonal variations, climate effect and fen-bog gradient, bound and free  
251 phenolic contents were significantly higher (ANOVA  $P < 0.001$ ) in top segments as compared  
252 to bottom segments (Fig. 2), except bound phenolics in the bog area ( $P = 0.16$ ). The two  
253 phenolics variables were also positively correlated, with respectively  $r = 0.38$  and  $0.37$  in the  
254 bog area (ANOVA,  $P < 0.01$ ) and  $r = 0.70$  and  $0.41$  in the fen area (ANOVA,  $P < 0.001$ ). The  
255 climate effect on bound phenolics resulted in a decrease of concentration of an average of  $0.4$   
256  $\text{mg g}^{-1}$  DM in the two sampling areas, particularly in spring and summer in top segments ( $P =$   
257  $0.04$  and  $0.02$ , respectively). The climate effect on free phenolics was essentially recorded in  
258 the fen area for both *Sphagnum* segments, with constantly lower concentrations in OTCs than  
259 in controls over the seasons (ANOVA,  $P = 0.001$ ) (Fig. 2), whereas the climate effect in the  
260 bog area was more rare.

261         In controls, seasonal variations of bound phenolics were recorded in top segments  
262 along the fen-bog gradient ( $P = 0.04$  and  $0.05$ , respectively) (Fig. 2a, b, c, d), especially from  
263 May to August with a significant decrease of an average of  $1.5 \text{ mg.g}^{-1}$  DM. In bottom

264 segments of controls, no significant seasonal variations of bound phenolics were recorded  
265 along the fen-bog gradient ( $P = 0.86$  and  $0.66$ , respectively), with an average of respectively  
266  $1.5 \text{ mg g}^{-1}$  DM in the bog area and  $1.0 \text{ mg g}^{-1}$  DM in the fen area. As for bound phenolics,  
267 seasonal variations of free phenolics in controls were recorded in top segments with a  
268 significant decrease in summer (from  $1.4$  to  $0.8 \text{ mg g}^{-1}$  DM in the two sampling areas;  $P <$   
269  $0.01$  and  $0.03$ , respectively). In bottom segments, no seasonal variations of free phenolics  
270 were recorded, with an average of  $0.8 \text{ mg g}^{-1}$  DM along the fen-bog gradient (Fig. 2e, f, g, h).  
271 In addition, a significant correlation was found between the decrease of phenolics (free and  
272 bound) and the decrease of *Sphagnum* moisture content in summer (ANOVA,  $P < 0.01$ ) in  
273 both segments in the bog area, and in top segments in the fen area.

274 In OTCs, the same seasonal variations as in controls were recorded in *Sphagnum*  
275 segments and for both phenolics along the fen-bog gradient ( $P < 0.05$  for all) (Fig. 2). As for  
276 controls, the same significant correlations were recorded between the decrease of phenolics  
277 (free and bound) and the decrease of *Sphagnum* moisture content in summer (ANOVA,  $P <$   
278  $0.05$ ).

279 Climate effect on culturable fungi-producing phenoloxidasases and enzymatic activity, and  
280 seasonal variations

281 Significant differences between top and bottom segments of *Sphagnum* were recorded with  
282 overall higher densities of fungi-producing phenoloxidasases and higher phenoloxidasase  
283 activities in bottom segments as compared to top segments in both sampling areas (ANOVA  
284  $P < 0.05$ ).

285 For densities of culturable fungi-producing phenoloxidasases, the climate effect was  
286 only significant in the fen area in top segments (ANOVA  $P = 0.03$ ), with a significant lower  
287 value in June in OTCs compared to control (Fig. 3a, b). Seasonal variations were recorded for

288 both *Sphagnum* segments in the fen and bog area, with a peak in June in controls ( $P < 0.05$ )  
289 (Fig. 3 a, b, c, d), while in OTCs this peak was only recorded in the bog area (Fig. 3c, d).  
290 Climate effects on phenoloxidase activity demonstrated equivocal results in the fen area,  
291 while phenoloxidase activity tended to be higher in OTCs in the bog area (Fig. 3e, g).

292         Significant positive correlations were also found between densities of fungi-producing  
293 phenoloxidases and extracellular phenoloxidase activities, in both sampling areas and both  
294 climate treatments (on average  $r = 0.40$ ; ANOVA,  $P < 0.05$ ). In parallel, significant negative  
295 correlations between free phenolic compounds and phenoloxidase activities were found for  
296 controls in the fen and bog areas when top and bottom *Sphagnum* segments were pooled (Fig.  
297 4a, b). The same tendency was recorded in OTCs, except in the bog area (Fig. 4b).  
298 Additionally, the combination of fungi and free phenols in a general linear model explained  
299 respectively 27.4% and 10.6% of the variability of phenoloxidase activity in controls, and  
300 29.6% and 0.6% in OTCs in the bog area (adjusted  $R^2$ ;  $P < 0.001$ ). For the fen area another  
301 patterns occurred since fungi and free phenolics explained respectively 13.7% and 9.8% of the  
302 variability of phenoloxidase activity in controls, and 11.3% and 25.8% in OTCs (adjusted  $R^2$ ;  
303  $P < 0.001$ ).

304 The phenol-phenoloxidase complex and its relation to abiotic variables

305 The contribution of the explanatory variables in the RDA (Table. 2) showed that time  
306 (months) has a major influence on the moss biochemical patterns. In bottom segments  
307 sampling time explained between 41% and 66% of the variation. In top moss segments,  
308 biplots of partial RDAs showed that *Sphagnum* related biochemical variables were influenced  
309 by climate treatment, as shown by the separation of control and OTC plots along the first  
310 RDA axis (Fig. 5a, c). Together, OTCs and *Sphagnum* moisture content explained 20.6%  
311 (fen) and 27.1% (bog) of the variation of biochemical factors ( $P < 0.05$ ) in top segments.

312 Variation partitioning and adjusted  $R^2$  showed that OTCs alone explained a higher variation in  
313 the fen area than in the bog area, whereas *Sphagnum* moisture content has higher influence in  
314 the bog than in the fen (Table 2). On the other hand, the biochemical descriptors showed a  
315 strong opposition between phenolics and warming treatment (OTC) in all biplots, while fungi  
316 appears linked to *Sphagnum* moisture content, particularly in top segments.

317 If we consider all samples together along the fen-bog gradient in the multiple factor  
318 analysis (Fig. 6), a clear pattern appeared, with a split into the three seasons (spring, summer  
319 and autumn) and within each partition a subdivision into fen and bog areas, each of these  
320 subdivisions being further divided into OTC and control plots. The RV-coefficients (Table 3)  
321 indicate strongest links between *Sphagnum* related biochemical variables, sampling area,  
322 climate warming and seasons, and between sampling area and physicochemical environment.

323

## 324 **Discussion**

325 Polyphenol/phenoloxidase interplay in *Sphagnum* mosses and along the fen-bog gradient

326 *Sphagnum* related biochemical factors quantified in this work yielded different results  
327 according to *Sphagnum* segments. Total phenolic content (free and bound) was higher in  
328 living top segments as compared to decaying bottom segments in both sampling areas. Such  
329 differences have been also observed in *S. fallax* under controlled conditions (Jassey *et al.*,  
330 2011b). This phenomenon is explained by a higher phenolic metabolism in capitulum than in  
331 lower part of the shoot, since *Sphagnum* capitula (top segments) constitute the living part of  
332 the moss where most of the metabolic processes occur, including the growth (Clymo &  
333 Hayward, 1982). The reduction of phenolics towards the lower part of the shoot was also  
334 accompanied by an increase of culturable fungi-producing phenoloxidases and of  
335 phenoloxidase activity, suggesting a higher degradation of recalcitrant phenolics in early

336 declining *Sphagnum* segments (Baldrian, 2006; Toberman *et al.*, 2010; Sinsabaugh & Follstad  
337 Shah, 2011). These results also pointed to the fact that at low concentrations free phenols may  
338 induce phenoloxidase activity, and inhibit the oxidation activity at high concentration  
339 (Sinsabaugh, 2010). Given that no clear correlation was found between fungi and free  
340 phenols, such vertical gradient also highlighted a possible direct inhibitory effect of free  
341 phenols on phenoloxidase activity (Wetzel, 1992; Freeman *et al.*, 2001a; Fenner *et al.*, 2005).

342 Our results likewise demonstrated a strong relationship between fungi and  
343 phenoloxidase activities. Phenoloxidase activity is essentially attributable to lignolytic fungi  
344 such as basidiomycetes (Criquet *et al.*, 2000; Thormann *et al.*, 2002; Baldrian, 2006). Fungal  
345 activity is known to be directly influenced by the supply of organic matter (Berg *et al.*, 1998;  
346 Criquet *et al.*, 2000). A study in the same experimental site demonstrated over the fen-bog  
347 gradient an increase of organic matter content in the upper 10 cm soil layer, which induced  
348 higher fungal activity (Delarue *et al.*, 2011). Thus, all of these findings emphasize that  
349 phenoloxidase activity was mainly controlled by fungi and secondarily by phenols.

350 Beside the differences between *Sphagnum* segments, different patterns of polyphenol  
351 content and phenoloxidase activities were recorded along the fen-bog gradient over the  
352 seasons. In particular, phenoloxidase activities were more intense in the bog area than in the  
353 fen area. Again, this result appeared linked to fungi. The abundance of vascular plants is  
354 higher in the bog area and supplies more easily decomposable organic matter, favouring  
355 fungal activity (Delarue *et al.*, 2011). A number of studies have demonstrated that fen and bog  
356 litters were characterized by distinct patterns of microfungal community, especially in the  
357 surface horizons (Thormann *et al.*, 2001, 2002, 2004; Thormann, 2006; Artz *et al.*, 2007).  
358 Thus, vegetation patchiness along the fen-bog gradient may directly affect fungal community  
359 composition, and indirectly phenoloxidase activity. In particular, the quality and quantity of  
360 plant-derived labile carbon resulting from vegetation succession may directly influence fungal



361 diversity, e.g. polymer- and recalcitrant polymer degraders (Thormann, 2006). On the other  
362 hand, the influence of free phenols on phenoloxidases was higher in the fen area than in the  
363 bog and this could be explained by qualitative differences of phenolics in *Sphagnum* along the  
364 gradient (Opelt *et al.*, 2007). When comparing phenolic content in *Sphagnum* from different  
365 ecological setting, Folin assay only gives a global tendency of phenolic variation, and not the  
366 quality of free phenols that may influence phenoloxidase activity. Such results clearly call for  
367 a detailed analysis of phenolic variation (e.g. phenolic acids or flavonoids).

368 Climate effect on polyphenols, phenoloxidases and their interactions along the fen-bog  
369 gradient

370 As described in previous studies (Dorrepaal *et al.*, 2004; Aerts, 2006), higher air temperatures  
371 induced higher evapotranspiration, which resulted in lower *Sphagnum* moisture content  
372 during summertime. Obviously, higher evapotranspiration also could have sometimes induced  
373 lower soil temperature by heat loss towards atmosphere and reduction of soil thermal  
374 conductivity, thus explaining the so-called marginal effect of OTCs on soil temperature  
375 (Dabros *et al.*, 2010). Despite contrasted effects of OTCs on air and soil temperature, a  
376 climate effect has been recorded on biochemical variables measured along *Sphagnum*  
377 segments.

378         Seasonal effects were predominant for the biochemical variation in *Sphagnum* carpet.  
379 However, multivariate analyses revealed a climate warming effect beyond the seasonal  
380 variations of *Sphagnum* biochemical related factors. As observed elsewhere (Aerts, 2006;  
381 Bragazza, 2008; Dabros and Fyles, 2010; Dabros *et al.*, 2010), the increase of air temperature  
382 associated with the reduction in rainfall led to heat waves, and the impact of these events was  
383 exacerbated in OTCs increasing drought in top-soil. Enhanced top-soil aeration as a result of  
384 water table drawdown and air temperature increase was recognized to influence

385 phenoxidase activity and polyphenols (Freeman *et al.*, 1993, 2001a, b; Toberman *et al.*,  
386 2008; Ellis *et al.*, 2009). As supported by current findings in peatlands (Pind *et al.*, 1994;  
387 Williams *et al.*, 2000; Freeman *et al.*, 2001a; Toberman *et al.*, 2008, 2010; Sinsabaugh, 2010),  
388 peat soil environmental factors (i.e. acidic pH, water table depth, and oxygen) mainly inhibit  
389 phenoxidase activity, explaining our weak variations of phenoxidases with climate  
390 warming.

391 In parallel, climate warming had greatest impact on the phenolic metabolism with a  
392 decrease of phenolics related to the decrease of *Sphagnum* moisture in OTCs and the increase  
393 of air temperatures. The level of total phenolic compounds tends to be lower in several boreal  
394 species under elevated temperatures (Veteli *et al.*, 2007). Such decrease may be explained by  
395 a diminution of carbon partitioning to phenolics (Herms & Mattson, 1992; Mattson *et al.*,  
396 2005). Elevated temperatures are recognized to induce better growth of *Sphagnum* species  
397 (Breeuwer *et al.*, 2008). It might well be that a trade-off between growth and differentiation  
398 (i.e. the production of carbon-based secondary metabolites such as phenols) occurred, with a  
399 potential diminution of carbon skeletons allocation to phenolics (Mattson *et al.*, 2005; Veteli  
400 *et al.*, 2007). Such results imply that any repeated significant decrease of phenolics through  
401 more intense and frequent heat waves – as predicted by climate scenarios (Meehl & Tabaldi,  
402 2004; Schär *et al.*, 2004; IPCC, 2007) – will probably lead to the opening of the enzymatic  
403 latch, as described by Freeman *et al.* (2001b).

404 Furthermore, our climate experiment demonstrated that climate warming has not had  
405 the same impact along the fen-bog gradient since a stronger decrease of polyphenols was  
406 recorded in the fen area. This decrease induced a switch between fungi and free phenols,  
407 leading to a reduction of the potential inhibitory effect of free phenols on phenoxidases.  
408 However, the decrease in the density of culturable fungi-producing phenoxidase during  
409 dryer periods could not compensate for the decrease of phenolics and lowering of their

410 inhibitory effect on phenoloxidase activity. Alternatively, or additionally, phenolics may also  
411 have inhibitory effects on other microbial activities with implication for the carbon cycle,  
412 such as hydrolase activity (Fenner *et al.*, 2005, 2007). Thus, the reduction of the inhibitory  
413 effect of free phenols could affect carbon cycling in the fen area through another  
414 microbial/polyphenols interplay (e.g. Jassey *et al.*, 2011a). In the bog area phenoloxidase  
415 activity remained the key factor influenced by climate treatment with a slight increase of  
416 activity in top segments, leading to potentially higher degradation of recalcitrant materials in  
417 surface horizons. In contrast to the fen area, it appeared that fungi mainly influenced  
418 phenoloxidases in OTCs, as shown by GLMs.

419         Although a slight increase of temperature induced by OTCs is not strong enough to  
420 significantly affect the decomposition rate of *Sphagnum* litter on short-time scale (Dabros *et*  
421 *al.*, 2010), our results demonstrated that already within a 7-month period key elements of the  
422 carbon cycle can be altered in surface horizons. Furthermore, our climate experiment  
423 highlights different responses of *Sphagnum* related biochemical variables along the fen-bog  
424 gradient. The main consequence is that not all the peatland habitats would respond similarly  
425 to climate forcing. Ultimately, our results suggest a destabilization of peatland ecosystems  
426 and reinforce the point that phenoloxidase/polyphenol interplay is especially critical to  
427 understanding the response of peatlands to climate change.

428

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438

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618



619 Table 1: Seasonal variations of environmental variables measured in controls and OTCs in the  
620 fen and bog sampling areas in Le Forbonnet mire (French Jura). *Letters* indicate significant  
621 seasonal variations ( $P < 0.05$ ). *Asterisks* indicate significant variations between controls and  
622 OTCs ( $P < 0.05$ ).

623 Table 2: Summary of RDA on *Sphagnum* related biochemical variables and environmental  
624 explanatory variables from Le Forbonnet mire (French Jura): fraction of variance explained  
625 and significance of individual variables taken alone. *Sph* moisture = *Sphagnum* moisture  
626 content; clim treat = climate treatment.

627 Table 3: RV-coefficients (RV) and corresponding *P*-values among the six groups of variables  
628 used in the Multiple factor analysis (MFA) of the entire data set split into 6 groups of  
629 variables describing *Sphagnum* biochemistry, environmental physical and chemical  
630 conditions, climate warming treatment, seasons, depth of moss segment and bog/fen areas .  
631 Significant coefficients are in bold.

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640 Figures:

641 Figure 1: Seasonal variations of *Sphagnum* moisture content in the two shoot segments (top  
642 and bottom) in controls and OTCs of the fen (a, b) and bog (c, d) areas. Mean  $\pm$  S.E. (n = 3).  
643 *Asterisk* indicates significant difference between controls and OTCs (ANOVA tests,  $P <$   
644 0.05).

645 Figure 2: Seasonal variations of bound (a, b, c, d) and free (e, f, g, h) phenolics in the two  
646 shoot segments (top and bottom) in controls and OTCs of the bog and fen areas. Mean  $\pm$  S.E.  
647 (n = 3). *Asterisk* indicates significant difference between controls and OTCs (ANOVA tests,  $P$   
648  $< 0.05$ ).

649 Figure 3: Seasonal variations of densities of fungi producing phenoloxidasases (a, b, c, d) and  
650 phenoloxidasase activities (e, f, g, h) in the two shoot segments (top and bottom) in controls and  
651 OTCs of the bog and fen areas. Mean  $\pm$  S.E. (n = 3). *Asterisk* indicates significant difference  
652 between controls and OTCs (ANOVA tests,  $P < 0.05$ ).

653 Figure 4: Correlations between free phenolics and phenoloxidasase activity for *Sphagnum*  
654 segments (top and bottom segments pooled) in controls and OTCs in the fen (a) and bog (b)  
655 areas.

656 Figure 5: Biplots of redundancy analyses (RDA) of biochemical data measured on *Sphagnum*  
657 mosses (free and bound phenolics, phenoloxidasases and fungi-producing phenoloxidasases) in  
658 top (a) and bottom (b) *Sphagnum* segments of the fen area, and in top (c) and bottom (d)  
659 segments of the bog area. Climate treatments are coded with open symbol for controls and  
660 with filled symbol for OTCs. Months are indicated next to the sample points by their number.  
661 Season effect has been removed by giving the variable months as covariable. Environmental  
662 variables are represented by vectors (arrows for quantitative or semi-quantitative variables):  
663 *Sph\_moist.*: *Sphagnum* moisture content; *Sph\_moist:OTC*: interactions between *Sphagnum*

664 moisture and OTCs. Biochemical variables are given with dotted arrows: F\_phen: free  
665 phenolics, B\_phen: bound phenolics; Phen\_oxid: phenoloxidase activity; Fungi: culturable  
666 fungi-producing phenoloxidase. Axes are significant ( $P < 0.05$ ), except for bottom segments.  
667 Axes 3 are never significant, with less than 1% of variance). *Grey ellipses* represent S.E. of  
668 site scores around the centroid of each treatment level.

669 Figure 6: Multiple factor analysis (MFA) samples biplot of the entire data set split into 7  
670 groups of variables describing *Sphagnum* biochemistry, environmental physical and chemical  
671 conditions, climate warming treatment, seasons and fen-bog areas. Biplot of axes 1 and 2  
672 (both significant at  $P = 0.001$ ) is given together with the result of a hierarchical agglomerative  
673 clustering (grey lines) obtained by the Ward method on the Euclidean distance matrix  
674 between MFA site scores, showing three main groups of sampling plots (circles = spring,  
675 squares = summer, triangles = autumn) and two sub-groups (white symbols = controls, black  
676 symbols = OTCs). Sampling areas are indicated with letters besides sampling plots (F: fen  
677 area; B: bog area).

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